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(57) Abstract

Amino acid sequences substantially identical to the C-terminal end of an α-S2 casein precursor are shown to act as growth promoters. Disclosed are sequences from Bovine α-S2 casein including the 9 C-terminal amino acids: LysValIleProTyrValArgTyrLeu. Also disclosed are foodstuffs and medicaments comprising the peptides of the invention and a method of producing same.

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DESCRIPTION

CASEIN FRAGMENTS HAVING GROWTH PROMOTING ACTIVITY

The present invention relates to growth promoters.

It has long been known that milk contains growth promoting activity for cells that is additional to its nutritional content. Thus, Epidermal Growth Factor (EGF) has been identified in human (Shing and Klagsbrun, 1984, Petrides, 1985), rat (Raaberg et al, 1990), swine (Tan et al 1990) and goat (Brown and Blakeley, 1983) milk.

Indeed the EGF present in rat milk has been shown to be significant for the normal development of pups (Oka et al 1983). EGF has not, however, been found in bovine milk (Read 1985). Instead insulin-like growth factor (IGF) I and II (Francis et al, 1986) and bovine colostrum growth factor (BCGF), which is structurally related to Platelet-derived Growth Factor (PDGF) (Shing and Klagsbrun, 1984, Brown and Blakeley, 1984), have been identified.

The applicant has surprisingly discovered that bovine milk contains growth promoting activity for rat mammary fibroblast cell line (Rama 27), which is not significantly stimulated by IGF or PDGF.

Furthermore, they have identified peptide sequences which elicit this growth promoting activity.

The invention relates to a peptide or a salt thereof comprising an amino acid sequence substantially identical to the C-terminal end of the α-S2 casein precursor.

According to a first aspect of the present invention there is provided the use of a peptide or a salt thereof comprising an amino acid sequence substantially identical to the C-terminal end of an α-S2 casein precursor, for the manufacture of a medicament or foodstuff for promoting growth.

Whilst whole casein protein shows no growth activity, the applicant has identified a number of peptides, derived from the C-terminal end of Bovine α-S2 casein, which elicit growth promoting activity.

Indeed, the applicant has shown this growth promoting activity to be present in at least peptides of 9 to 31 amino acids in length which have been derived from the C-terminal end of Bovine α-S2 casein. It is reasonable to hypothesise that the natural sequence responsible for the growth promoting activity is the sequence comprising the last 9 amino acids of the C-terminal end or an even shorter sequence from within the nine amino acid sequence, possibly an 8 or 7 amino acid sequence. Indeed, it may be as short as a 3 amino acid sequence.

The bovine α-S2 casein precursor is characterised

in that it has an amino acid sequence:

[CAS2_BOVIN] ALPHA-S2 CASEIN PRECURSOR.
SEQUENCE
MKFFIFTCLL AVALAKNTME RVSSSEESII SQETYKQEKN MAINPSKENL CSTFCKEVVR
NANEEEYSIG SSSEESAEVA TEEVKITVDD KHYQKALNEI NQFYQKFPQY LQYLYQGPIV
LNPWDQVKRN AVPITPTLNR EQLSTSEENS KKTVDMESTE VFTKKTKLTE EEKNRLNFLK
KISQRYQKFA LPQYLKTVYQ HQKAMKPWIQ PKTKVIPYVR YL

In three letter codes this translates to:

[CAS2_BOVIN] ALPHA-S2 CASE IN PRECURSOR.
SEQUENCE

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaLeuAlaLeuAsnThrMetGlu

HisValSerSerSerGluGluSerIleIle
SerGlnGluThrTyrLysGlnGluLysAsn

MetAlaIleAsnProSerLysGluAsnLeu
CysSerThrPheCysLysGluValValArg

AsnAlaAsnGluGluGluTyrSerIleGly
SerSerSerGluGluSerAlaGluValAla

ThrGluGluValLysIleThrValAspAsp
LysHisTyrGlnLysAlaLeuAsnGluIle

AsnGlnPheTyrGlnLysPheProGlnTyr
LeuGlnTyrLeuTyrGlnGlyProIleVal

LeuAsnProTrpAspGlnValLysArgAsn
AlaValProIleThrProThrLeuAsnArg

GluGlnLeuSerThrSerGluGluAsnSer
LysLysThrValAspMetGluSerThrGlu

ValPheThrLysLysThrLysLeuThrGlu
GluGluLysAsnArgLeuAsnPheLeuLys

LysIleSerGlnArgTyrGlnLysPheAla
LeuProGlnTyrLeuLysThrValTyrGln

HisGlnLysAlaMetLysProTrpIleGln
ProLysThrLysValIleProTyrValArg

TyrLeu

The applicant has found that short peptide sequences incorporating the C-terminal sequence -LysValIleProTyrValArgTyrLeu show growth promoting activity.

According to a second aspect of the present invention there is provided a growth factor comprising the amino acid sequence -LysValIleProTyrValArgTyrLeu

Furthermore, comparison of, for example, the last 20 amino acids of the C-terminal sequence for bovine α-S2 casein with those for goat, and sheep shows a high degree of homology as does to a lesser extent the C-terminal amino acid sequence of rabbit and pig α-S2 casein

The sequences for these are set out below.

[CAS2_CAPHI] ALPHA-S2 CASEIN PRECURSOR (ALPHA-S2-CN).
SEQUENCE
MKFFIFTCLL AVALAKHKME HVSSSEEPIN IFQEIYKQEK NMAIHPRKEK LCTTSCEEVV
RNANEEEYSI RSSSEESAEV APEEIKITVD DKHYQKALNE INQFYQKFPQ YLQYPYQGPI
VLNPWDQVKR NAGPFTPTVN REQLSTSEEN SKKTIDMEST EVFTKKTKLT EEEKNRLNFL
KKISQYYQKF AWPQYLKTVD QEQKAMKPWT QPKTNAIPYV RYL

>pir|S33881|S33881 alphas2-casein B - goat

MKFFIFTCLL AVALAKHKME HVSSSEEPIN IFQEIYKQEK NMAIHPRKEK LCTTSCEEVV
RNANEEEYSI RSSSEESAKV APEEIKITVD DKHYQKALNE INQFYQKFPQ YLQYPYQGPI
VLNPWDQVKR NAGPFTPTVN REQLSTSEEN SKKTIDMEST EVFTKKTKLT EEEKNRLNFL
KKISQYYQKF AWPQYLKTVD QEQKAMKPWT QPKTNAIPYV RYL 223

>gp|S74171|S74171_1 alpha s2-casein C [Capra hircus]

MKFFIFTCLL AVALAKHKME HVSSSEEPIN IFQEIYKQEK NMAIHPRKEK LCTTSCEEVV
RNANEEEYSI RSSSEESAEV APEEIKITVD DKHYQKALNE INQFYQKFPQ YLQYPYQGPI
VLNPWDQVKR NAGPFTPTVN REQLSTSEEN SKKTIDMEST EVFTKKTKLT EEEKNRLNFL
KIISQYYQKF AWPQYLKTVD QEQKAMKPWT QPKTNAIPYV RYL 223

>pir|S39776|S39776 alpha-S2-casein form b precursor - rabbit
>gp|X76909|OCPAS2BCS_1 pre-alpha S2b casein (AA -15 to 167)
[Oryctolagus cuniculus]

MKFFIFTCLL AVALAKPKIE QSSSEETIAV SQEVSPNLEN ICSTACEEPI KNINEVEYVE
VPTEIKDQEF YQKVNLLQYL QALYQYPTVM DPWTRAETKA IPFIRTMQYK QEKDATKHTS
QKTELTEEEK AFLKYLDEMK QYYQKFVFPQ YLKNAHHFQK TMNPWNHVKT IIYQSVPTL 179

[CAS2_SHEEP] ALPHA-S2 CASEIN PRECURSOR.
SEQUENCE
MKFFIFTCLL AVALAKHKME HVSSSEEPIN ISQEIYKQEK NMAIHPRKEK LCTTSCEEVV
RNADEEEYSI RSSSEESAEV APEEVKITVD DKHYQKALNE INQFYQKFPQ YLQYLYQGPI
VLNPWDQVKR NAGPFTPTVN REQLSTSEEN SAKTIDMEST EVFTKKTKLT EEEKNRLNFL
KKISQYYQKF AWPQYLKTVD QEQKAMKPWT QPKTNAIPYV RYL

[CAS2_PIG] ALPHA-S2 CASEIN PRECURSOR.
SEQUENCE
MKFFIFTCLL AVAFAKHEME HVSSSEESIN ISQEKYKQEK NVINHPSKED ICATSCEEAV
RNIKEVGYAS SSSSEESVDI PAENVKVTVE DKHYLKQLEK ISQFYQKFPQ YLQALYQAQI
VMNPWDQTKT SAYPFIPTVI QSGEELSTSE EPVSSSQEEN TKTVDMESME EFTKKTELTE
EEKNRIKFLN KIKQYYQKFT WPQYIKTVEQ KQKAMKPWNH IKTNSYQIIP NLRYF

In three letter code these translate to:

[CAS2_CAPH1] ALPHA-S2 CASEIN PRECURSOR (ALPHA-S2-CN).
SEQUENCE

MetLysPheIlePhePheThrCysLeuLeu
AlaValAlaLeuAlaLysHisLysMetGlu

HisValSerSerSerGlyGlyProIleAsn
IlePheGlnGluIleTyrLysGlnGluLys

AsnMetAlaIleHisProArgLysGluLys
LeuCysThrThrSerCysGluGluValVal

ArgAsnAlaAsnGluGluGluTyrSerIle
ArgSerSerSerGluGluSerAlaGluVal

AlaProGluGluIleLysIleThrValAsp
AspLysHisTyrGlnLysAlaLeuAsnGlu

IleAsnGlnPheTyrGlnLysPheProGln
TyrLeuGlnTyrProTyrGlnGlyProIle

ValLeuAsnProTrpAspGlnValLysArg
AsnAlaGlyProPheThrProThrValAsn

ArgGluGlnLeuSerThrSerGluGluAsn
SerLysLysThrIleAspMetGluSerThr

GluValPheThrLysLysThrLysLeuThr
GluGluGluLysAsnArgLeuAsnPheLeu

LysLysIleSerGlnTyrTyrGlnLysPhe
AlaTrpProGlnTyrLeuLysThrValAsp

GlnHisGlnLysAlaMetLysProTrpThr
GlnProLysThrAsnAlaIleProTyrVal

ArgTyrLeu

>pir/S33881/S33881 alpha S2-casein E goat

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaLeuAlaLysHisLysMetGlu
HisValSerSerSerGluGluProIleAsn
IlePheGlnGluIleTyrLysGlnGluLys
AsnMetAlaIleHisProArgLysGluLys
LeuCysThrThrSerCysGluGluValVal
ArgAsnAlaAsnGluGluGluTyrSerIle
ArgSerSerSerGluGluSerAlaLysVal
AlaProGluGluIleLysIleThrValAsp
AspLysHisTyrGlnLysAlaLeuAsnGlu
IleAsnGlnPheTyrGlnLysPheProGln
TyrLeuGlnTyrProTyrGlnGlyProIle
ValLeuAsnProTrpAspGlnValLysArg
AsnAlaGlyProPheThrProThrValAsn
ArgGluGlnLeuSerThrSerGluGluAsn
SerLysLysThrIleAspMetGluSerThr
GluValPheThrLysLysThrLysLeuThr
GluGluGluLysAsnArgLeuAsnPheLeu
LysLysIleSerGlnTyrTyrGlnLysPhe
AlaTrpProGlnTyrLeuLysThrValAsp
GlnHisGlnLysAlaMetLysProTrpThr
GlnProLysThrAsnAlaIleProTyrVal

ArgTyrLeu 223

>pir/S74171/S74171 1 alpha S2-casein C [Capra hircus]

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaLeuAlaLysHisLysMetGlu
HisValSerSerSerGluGluProLIeAsn
IlePheGlnGluIleTyrLysGlnGluLys
AsnMetAlaIleHisProArgLysGluLys
LeuCysThrThrSerCysGluGluValVal
ArgAsnAlaAsnGluGluGluTyrSerIle
ArgSerSerSerGluGluSerAlaGluVal
AlaProGluGluIleLysIleThrValAsp
AspLysHisTyrGlnLysAlaLeuAsnGlu
IleAsnGlnPheTyrGlnLysPheProGln
TyrLeuGlnTyrProTyrGlnGlyProIle
ValLeuAsnProTrpAspGlnValLysArg
AsnAlaGlyProPheThrProThrValAsn
ArgGluGlnLeuSerThrSerGluGluAsn
SerLysLysThrIleAspMetGluSerThr
GluValPheThrLysLysThrLysLeuThr
GluGluGluLysAsnArgLeuAsnPheLeu
LysIleIleSerGlnTyrTyrGlnLysPhe
AlaTrpProGlnTyrLeuLysThrValAsp
GlnHisGlnLysAlaMetLysProTrpThr
GlnProLysThrAsnAlaIleProTyrVal
ArgTyrLeu 223

>pir/S39776/S39776 alpha-S2- Casein form b precursor - rabbit
>gp/X76909/OCPAS2BCS 1 pre-alpha S"b casein (AA -15 to 167)
[Oryctolagus cuniculus]

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaLeuAlaLysProLysIleGlu
GlnSerSerSerGluGluThrIleAlaVal
SerGlnGluValSerProAsnLeuGluAsn
IleCysSerThrAlaCysGluGluProIle
LysAsnIleAsnGluValGluTyrValGlu
ValProThrGluIleLysAspGlnGluPhe
TyrGlnLysValAsnLeuLeuGlnTyrLeu
GlnAlaLeuTyrGlnTyrProThrValMet
AspProTrpThrArgAlaGluThrLysAla
IleProPheIleArgThrMetGlnTyrLys
GlnGluLysAspAlaThrLysHisThrSer
GlnLysThrGluLeuThrGluGluGluLys
AlaPheLeuLysTyrLeuAspGluMetLys
GlnTyrTyrGlnLysPheValPheProGln
TyrLeuLysAsnAlaHisHisPheGlnLys
ThrMetAsnProTrpAsnHisValLysThr
IleIleTyrGlnSerValProThrLeu
179

[CAS2 SHEEP] ALPHA -S2 CASEIN PRECURSOR SEQUENCE.

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaLeuAlaLysHisLysMetGlu
HisValSerSerSerGluGluProIleAsn
IleSerGlnGluLIeTyrLysGlnGluLys
AsnMetAlaIleHisProArgLysGluLys
LeuCysThrThrSerCysGluGluValVal
ArgAsnAlaAspGluGluGluTyrSerIle
ArgSerSerSerGluGluSerAlaGluVal
AlaProGluGluValLysLIeThrValAsp
AspLysHisTyrGlnLysAlaLeuAsnGlu
IleAsnGlnPheTyrGlnLysPheProGln
TyrLeuGlnTyrLeuTyrGlnGlyProIle
ValLeuAsnProTrpAspGlnValLysArg
AsnAlaGlyProPheThrProThrValAsn
ArgGluGlnLeuSerThrSerGluGluAsn
SerLysLysThrIleAspMetGluSerThr
GluValPheThrLysLysThrLysLeuThr
GluGluGluLysAsnArgLeuAsnPheLeu
LysLysIleSerGlnTyrTyrGlnLysPhe
AlaTrpProGlnTyrLeuLysThrValAsp
GlnHisGlnLysAlaMetLysProTrpThr
GlnProLysThrAsnAlaIleProTyrVal
ArgTyrLeu

[CAS2 PIG] ALPHA-S2 CASEIN PRECURSOR.
SEQUENCE

MetLysPhePheIlePheThrCysLeuLeu
AlaValAlaPheAlaLysHisGluMetGlu
HisValSerSerSErGluGluSerIleAsp
IleSerGlnGluLysTyrLysGlnGluLys
AsnValIleAsnHisProSerLysGluAsp
IleCysAlaThrSerCysGluGluAlaVal
ArgAsnIleLysGluValGluTyrAlaSer
SerSerSerSerGluGluSerValAspIle
ProAlaGluAsnValLysValThrValGlu
AspLysHisTyrLeuLysGlnLeuGluLys
IleSerGlnPheTyrGlnLysPheProGln
TyrLeuGlnAlaLeuTyrGlnAlaGlnIle
ValMetAsnProTrpAspGlnThrLysThr
SerAlaTyrProPheIleProThrValIle
GlnSerGlyGluGluLeuSerThrSerGlu
GluProValSerSerSerGlnGluGluAsn
ThrLysThrValAspMetGluSerMetGlu
GluPheThrLysLysThrGluLeuThrGlu
GluGluLysAsnArgLIeLysPheLeuAsn
LysLIeLysGlnTyrTyrGlnLysPheThr
TrpProGlnTyrIleLysThrValHisGln
LysGlnLysAlaMetLysProTrpAsnHis
IleLysThrAsnSerTyrGlnIleIlePro
AsnLeuArgTyrPhe

It will be apparent from this that the C-terminal sequence can vary from species to species and that consequently whilst the preferred sequences comprise those derived from the C-terminal end of the bovine α-S2 casein those of the other species might be used.

Furthermore, due to the similar nature of some amino acids it is possible that minor substitutions may have little effect on the functioning of the sequence.

Thus, for example, Leucine, isoleucine and valine may be interchanged. Tyrosine and phenylalanine may be interchanged, and arginine and lysine may be interchanged

The significance of the discovery is that a peptide supplement which can promote growth can be added to food or drink products, for both human or animal consumption.

According to a further aspect of the present invention there is provided a food or drink product comprising a peptide or salt thereof of the invention.

Preferably the food or drink product is an infant formula or an animal feed. It may be in liquid or powder form.

Whilst it is possible to synthetically produce peptides according to the present invention it would be desirable to produce the peptide in situ from cows

milk.

According to a further aspect of the present invention milk is treated with an enzyme to break the casein in the milk into smaller fragments containing the active peptide or a salt thereof of the invention.

Preferably the enzyme is a protease and more particularly one which cleaves lysine cross-bonds. More preferably still it is plasmin or trypsin.

The invention will be further described by way of example only with reference to the following examples:

EXAMPLE 1

The growth promoting activity of different milk types was determined by precipitating caseins and assaying the supernatants for their ability to stimulate the incorporation of [3H] thymidine into the DNA of Rama 27 cells by known methodology (Smith et al, 1984).

The results of the tests are illustrated in Fig 1. which shows the growth-promoting activity of different milk types. Three sorts of commercial milks were acidified to precipitate the caseins and assayed for their growth promoting activity. The greatest activity was found in semi-skimmed milk. SDM (step down medium) represents the negative control and FCS (foetal calf serum) represents the positive control.

EXAMPLE 2

5 litres of semi-skimmed milk was made to pH 3.0 with HCl and left for 2 hours at 4°C. It was centrifuged in a Sorvall RC5B centrifuge at 9000 rpm in a GS3 rotor for 40 min, and the supernatant (approximately 3.6 litres) was poured through glass wool to remove fat. Solid $(NH_4)_2SO_4$ was added slowly to the supernatant with stirring at 4°C to a concentration of 22% (w/v), and was left for 2 hours at 4°C without stirring. Precipitated protein was removed by centrifugation as above. To the supernatant was added further $(NH_4)_2SO_4$ to a concentration of 35% (w/v) and the precipitate recovered as above. The precipitate was redissolved in 1600ml distilled water and dialyzed against running tap water overnight, then against 20mM NaH_2PO_4,pH6.0, for 8 hours.

The active fractions were obtained using a series of chromatographic techniques as outlined in (i) to (iv) below:

(i) The active fraction prepared as above was subjected to CM-Sepharose chromatography. It was added to a column of CM-Sepharose (10cm x 5cm id, Pharmacia) that had been pre-equilibrated with 20mM Sodium phosphate buffer pH6.0. After loading, the

column was washed with 500ml of 50mM NaCl in the same buffer. Protein was eluted with a 1500ml linear gradient of 0.1 to 0.7M NaCl in 20mM sodium phosphate buffer pH 6.0. The bioactive fractions eluted at 0.28M NaCl and approximately 0.4M NaCl - see Fig. 2. In Fig 2 the upper panel shows the absorbance of the protein at 280nm and the lower panel shows the activity (The incorporation of ^{3}H- thymidine into DNA). The sample was from material precipitating between 22 to 35% $(NH_4)_2SO_4$. After being redissolved and dialyzed it was loaded into the column (10 cm x 5 cm) with 0.05 M NaCl in 20mM NaH_2PO_4, pH 6.0. The eluting gradient was 0.1-0.7 M NaCl in 20 mM NaH_2PO_4,pH6. The flowrate was 5ml/min, the fraction size was 25 ml each. Two activities eluted at 0.28 M NaCl and 0.34-0.45 M NaCl respectively. The high absorbance at 280 nm at the beginning of the trace indicates the amount of unbound protein. The fraction-eluted at 0.28 M NaCl was used for further purification.

(ii) The active fractions from the above separation were subjected to hydrophobic interaction chromatography. It was made 3.7M with NaCl in 20mM NaH_2PO_4, pH6.5, and applied to a butyl Sepharose column (8.6 cm x 2.5 cm id) that had been pre-equilibrated with 4M NaCl in 20mM NaH_2PO_4,pH6.5.

Protein was eluted with a decreasing gradient of NaCl as indicated in Fig 3. In Fig. 3 the upper panel shows the absorbance of the protein at 280 nm and the lower panel shows the activity (The incorporation of ^{3}H-thymidine into DNA). The sample was from the early activity after CM-Sepharose chromatography. The column (2.5 cm x 8.6 cm, butyl bonded Sepharose) had been equilibrated with 4 M NaCl in 20 mM NaH_2PO_4, pH 6.5. The flowrate was 3.5 ml/min and fraction size was 3.5 ml. The activity eluted at 1.6 M NaCl, just before the major protein peak.

(iii)The active fractions from the hydrophobic interaction column were subjected to Reversed Phased HPLC-1 chromatography. It was applied in 8 batches to a butyl reversed phase column (Brownlee, 300A pore size, 7μm particle size, 25cm x 4.6mm id) that had been pre-equilibrated with 0.1% TFA. After washing the column with 0.1% TFA, protein was eluted with a gradient of acetonitrile (far uv grade, Rathburns, Walkerburn, Scotland) as indicated in Fig 4. In Fig. 4 the upper panel shows the absorbance of the protein at 214 nm and the lower panel shows the activity (The incorporation of ^{3}H-thymidine into DNA). The sample was from the activity after hydrophobic interaction chromatography. The column (250 cm x 4.6 mm, C4) had been equilibrated

with 0.1% TFA. The flow rate was 0.7 ml/min and fraction size was 0.7 ml. The eluting gradient was 10 to 30% acetonitrile in 0.1% TFA in 30 min. The activity eluted at 23% acetonitrile.

(iv) The active fractions were then subjected to reversed phase HPLC-2 chromatography. The mitogenic fractions from all 8 batches of the above reversed phase chromatograms were pooled and concentrated on a centrifugal drier to a total volume of 100µl. This concentrated material was loaded onto a C18 reversed phase column (ODS ultrasphere, Beckman) which had been pre-equilibrated with 0.1% TFA, and was eluted with a shallow gradient of 20 to 40% acetonitrile, 0.1% TFA over 45 min, at a flow rate of 0.2ml/min. Absorbance was monitored at 214nm, and material from each peak of absorbance was collected separately by hand - see Fig 5. In Fig. 5 the upper panel shows the absorbance of the protein at 214 nm and the lower panel shows the activity (The incorporation of ^{3}H-thymidine into DNA). The sample was from the activity after reversed phase HPLC-1. The column (ODS) had been equilibrated with 0.1% TFA. The flowrate was 0.2 ml/min. Each absorption peak at 214 nm was collected manually. The eluting gradient was 20 to 40% acetonitrile in 0.1% TFA in 45 min. The peaks A,B,C (arrows) were all active.

The purified proteins (peaks A,B,C) obtained in step (iv) were then analysed.

Protein content was measured by the binding of Coomassie Blue according to the Bio-Rad protocol, using bovine gamma globulin as standard. Peptide quantification of fractions separated by HPLC was by their absorbance at 214nm, using cytochrome c and lysozyme as standards.

The protein fractions A,B,C, of the casein digest where assayed for their ability to stimulate the incorporation of [3H] thymidine into the DNA of Rama 27 cells exactly as described previously.

The results are illustrated in Table 1 which shows the growth promoting activity of progressively purified fractions of α-S2 casein.

The peptides from the peaks B and C of reversed phase HPLC-2 were then sequenced. They were found to be a nested series of sequences of 5 peptides. They corresponded to the C-terminus of bovine α-S2 casein. The peak C was solely ThrLysValIleProTyrValArgTyrLeu, the other sequences were from peak B.

The sequences of the peaks are identified below:

Sequence 1 LysValIleProTyrValArgTyrLeu (peak B)

Sequence 2 ThrLysValIleProTyrValArgTyrLeu (peak C)

Sequence 3 LysThrLysValIleProTyrValArgTyrLeu (peak B)

Sequence 4

AlaMetLysProTrpIIeGlnProLysThrLysValIIeProTyrValArgTyrLeu (peak B)

Sequence 5

ProGlnTyrLeuLysThrValTyrGlnHisGlnLysAlaMetLysProTrpIIeGlnPro LysThrLysValIIeProTyrValArgTyrLeu (peak B)

To ascertain that the activity was not due to impurities identical peptide sequences were synthesized on a Milligen/Biosearch 9050 peptide synthesizer (Millipore, Watford) using Fmoc chemistry and pentafluorophenyl esters according to the standard protocol.

Of these initially only LysValIleProTyrValArgTyrLeu showed bioactivity, but after storage in PBS all the peptides acquired a low level of mitogenicity. The activity of LysValIIeProTyrValArgTyrLeu was substantially increased when maintained at alkaline pH. By way of contrast alpha-casein was inactive in the mitogenic assay. On digestion with trypsin, activity in the assay was generated, which was separable by reversed phase HPLC from that due to trypsin itself.

The example described herein demonstrates that the growth factor activity of milk is largely due to C-terminal fragments of α-S2 casein.

Given the activity of the peptide it is expected

that the addition of from 0.1μg to 10μg, more particularly about 1 μg of peptide to 250g of feed or drink will provide good growth promotion activity.

However, in order to maintain the activity the synthetic peptides should be stored in alkaline conditions, preferably at about pH 13.

SEQUENCE LISTING

SEQUENCE I.D. No 1

LENGTH: 9 amino acids

TYPE: Amino acid

SEQUENCE: LysValIleProTyrValArgTyrLeu

SEQUENCE I.D. No 2

LENGTH: 10 amino acids

TYPE: Amino acids

SEQUENCE: ThrLysValIleProTyrValArgTyrLeu

SEQUENCE I.D. No 3

LENGTH: 11 amino acids

TYPE: Amino acids

SEQUENCE: LysThrLysValIleProTyrValArgTyrLeu

SEQUENCE I.D. No 4

LENGTH: 19 amino acids

TYPE: Amino acids

SEQUENCE:

AlaMetLysProTrpIIeGlnProLysThrLysValIIeProTyrValArgTyrLeu

SEQUENCE I.D. No 5

LENGTH: 31 amino acids

TYPE: Amino acids

SEQUENCE:

ProGlnTyrLeuLysThrValTyrGlnHisGlnLysAlaMetLysProTrpIIeGlnPro

LysThrLysValIIeProTyrValArgTyrLeu

CLAIMS

1. Use of a peptide or a salt thereof comprising an amino acid sequence substantially identical to the C-terminal end of an α-S2 casein precursor, for the manufacture of a medicament or foodstuff for promoting growth.

2. Use of a peptide as claimed in claim 1, wherein the peptide is derived from bovine, goat, sheep, rabbit or pig α-S2 casein or is a synthesised equivalent or homologue thereof.

3. Use of a peptide as claimed in claim 2, wherein the peptide is derived from bovine α-S2 casein or is a synthesised equivalent or homologue thereof.

4. Use of a peptide as claimed in any of the preceding claims, in which the peptide comprises from 9 to 31 amino acids.

5. Use of a peptide as claimed in any of the preceding claims, in which the peptide comprises 9 amino acids.

6. Use of a peptide as claimed in any of the preceding claims comprising the amino acid sequence:

LysValIleProTyrValArgTyrLeu

or a homologue thereof.

7. Use of a peptide as claimed in any of claims 2 to 6, in which the homologues comprise peptides in

which:

i) one or more of the amino acids Leu, Ile and Val are replaced by one another;

ii) one or more of the amino acids Tyr and Phe are replaced by one another; and/or

iii) one or more of the amino acids Arg and Lys are replaced by one another.

8. Use of a peptide as claimed in any of claims 1 to 7, in which the peptide has the sequence: LysValIleProTyrValArgTyrLeu.

9. Use of a peptide as claimed in any of claims 1 to 7 in which the peptide has the sequence: ThrLysValIleProTyrValArgTyrLeu.

10. Use of a peptide as claimed in any of claims 1 to 7 in which the peptide has the sequence: LysThrLysValIleProTyrValArgTyrLeu.

11. Use of a peptide as claimed in any of claims 1 to 7 in which the peptide has the sequence: AlaMetLysProTrpIleGlnProLysThrLysValIleProTyrValArgTyrLeu.

12. Use of a peptide as claimed in any of claims 1 to 7 in which the peptide have the sequence: ProGlnTyrLeuLysThrValTyrGlnHisGlnLysAlaMetLysProTrpIleGlnPro LysThrLysValIleProTyrValArgTyrLeu.

13. Use of a peptide as claimed in any of the preceding claims in which foodstuff is an infant formula or an animal feed.

14. Use of a peptide as claimed in any of the preceding claims in which the medicament or foodstuff is a liquid or powder.

15. Use of a peptide as claimed in any of the preceding claims, in which the medicament or foodstuff comprises whole milk or semi-skimmed milk.

16. Use of a peptide as claimed in any of the preceding claims, in which the medicament or foodstuff has an alkaline pH.

17. Use of a peptide as claimed in any of the preceding claims, in which the peptide is present in an effective amount.

18. Use of a peptide as claimed in claim 17, wherein the effective amount is 0.1 to 10μg to 250g of medicament or foodstuff.

19. A food or drink product comprising a peptide or a salt thereof comprising an amino acid sequence substantially identical to the C-terminal end of an α-S2 casein precursor.

20. A method of producing a medicament or foodstuff comprising a growth promoting peptide comprises treating milk with an enzyme to break milk casein present in the milk into one or more peptides comprising an amino acid sequence substantially identical to the C-terminal end of the α-S2 casein precursor.

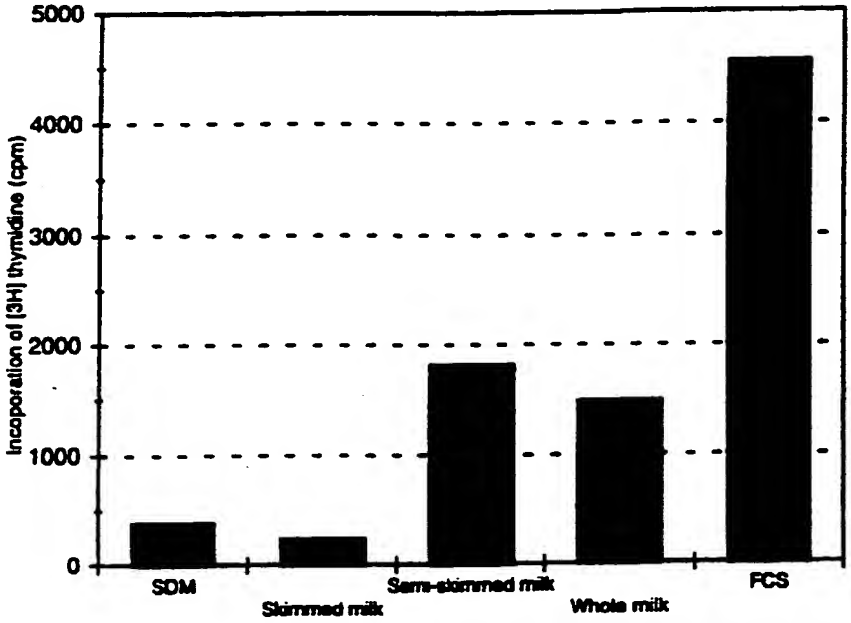

5000
4000
3000
2000
1000
0
Incorporation of [3H] thymidine (cpm)
SDM
Skimmed milk
Semi-skimmed milk
Whole milk
FCS

FIG. 1

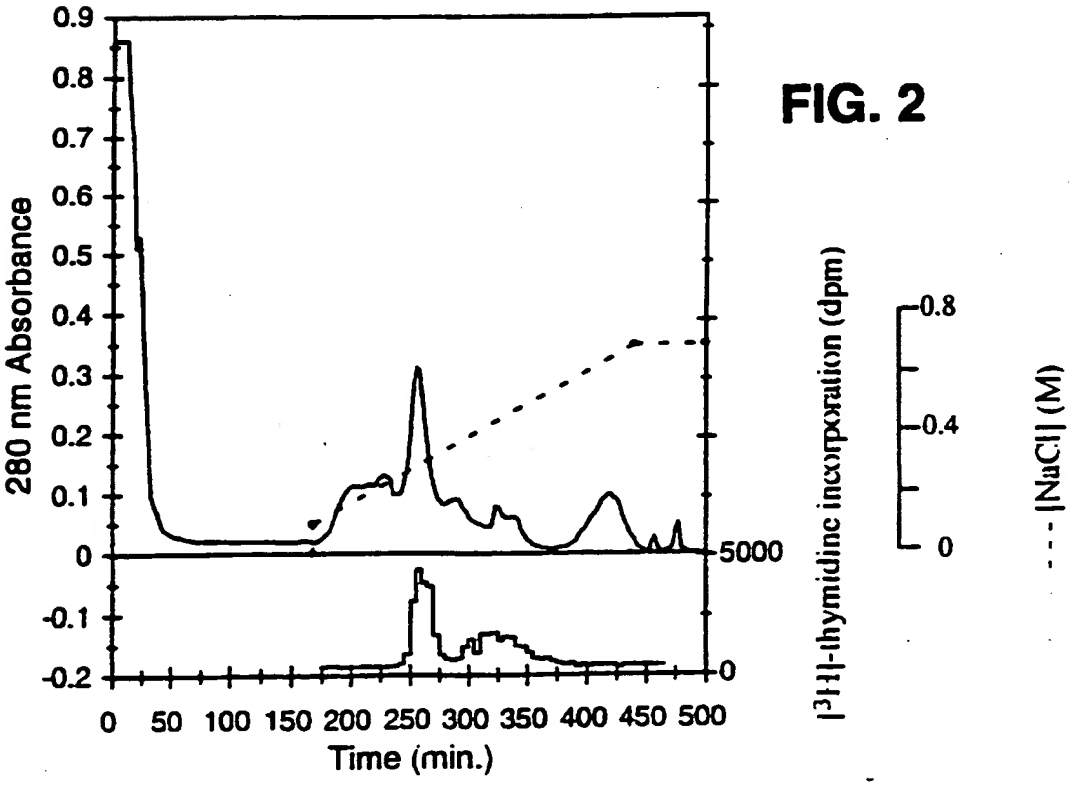

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
280 nm Absorbance
5000
0
0 50 100 150 200 250 300 350 400 450 500
Time (min.)
[3H]-thymidine incorporation (dpm)
0.8
0.4
0
--- [NaCl] (M)

FIG. 2

FIG. 3

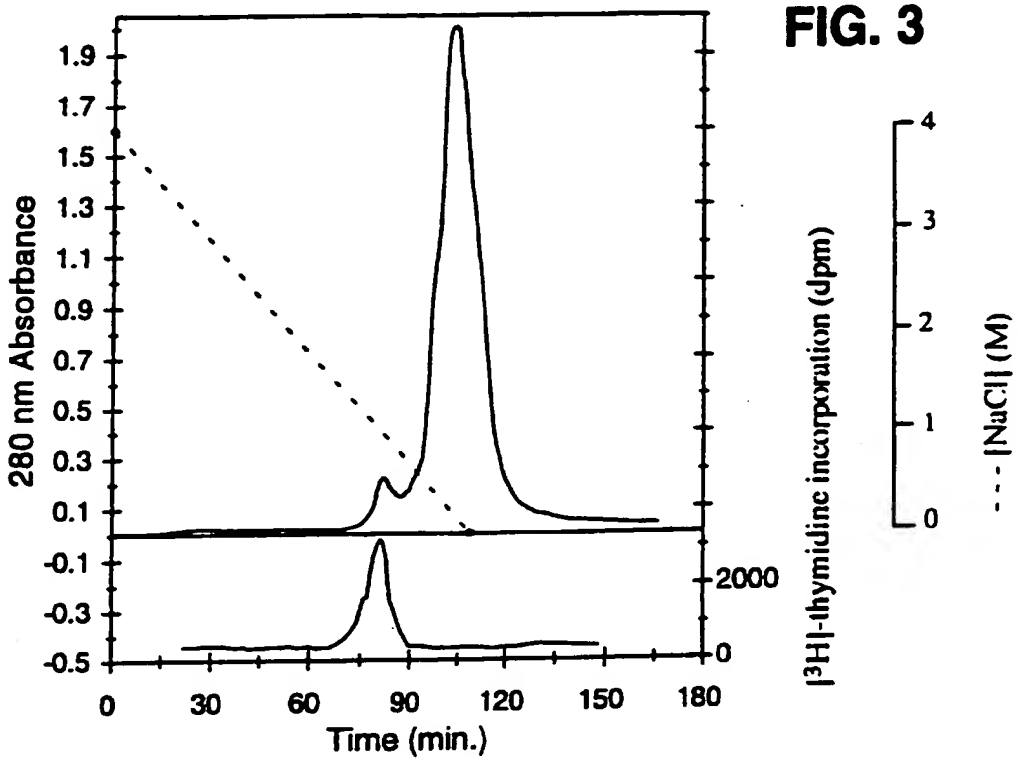
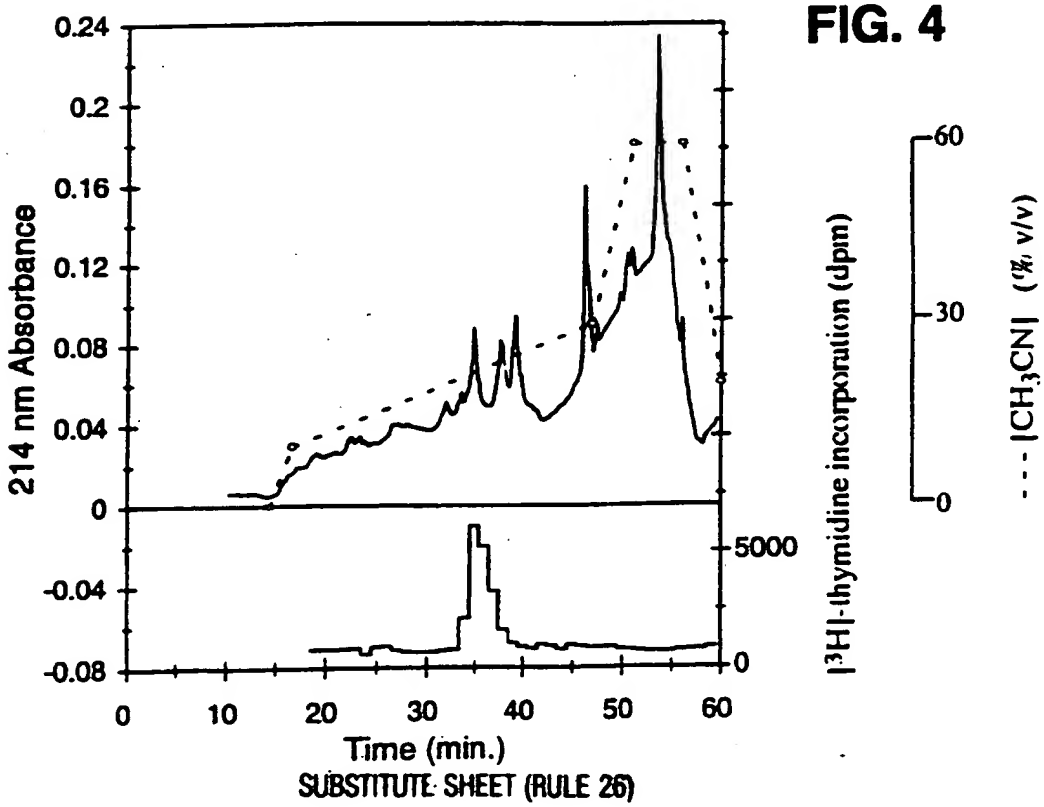

280 nm Absorbance
1.9
1.7
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
-0.1
-0.3
-0.5
2000
0
0
30
60
90
120
150
180
Time (min.)
[3H]-thymidine incorporation (dpm)
4
3
2
1
0
--- [NaCl] (M)

FIG. 4


214 nm Absorbance
0.24
0.2
0.16
0.12
0.08
0.04
0
-0.04
-0.08
5000
0
0
10
20
30
40
50
60
Time (min.)
[3H]-thymidine incorporation (dpm)
60
30
0
--- [CH3CN] (% v/v)

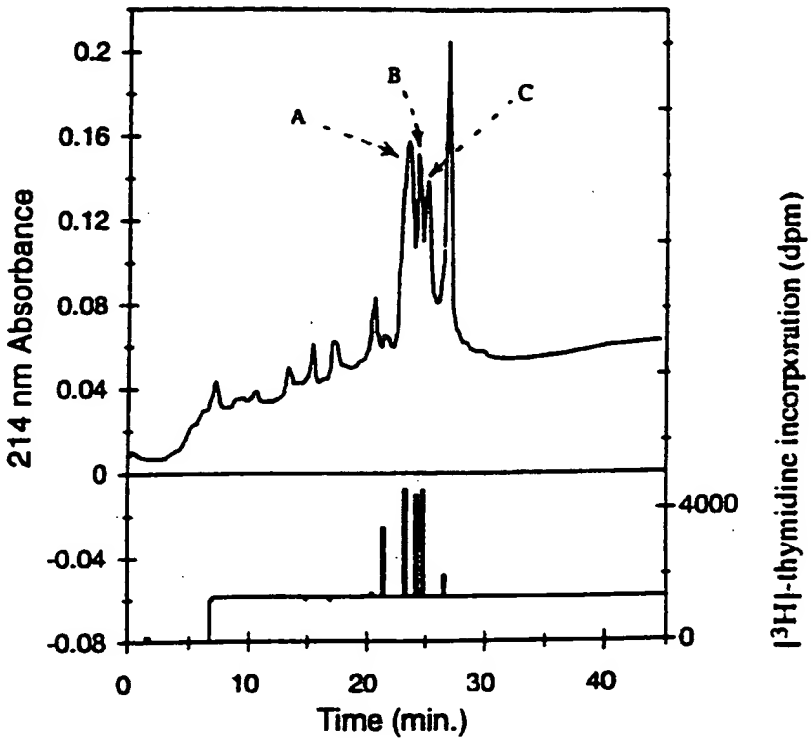

0.2
0.16
0.12
0.08
0.04
0
-0.04
-0.08
214 nm Absorbance
A
B
C
4000
0
[3H]-thymidine incorporation (dpm)
0
10
20
30
40
Time (min.)

FIG. 5

Table 1. Partial purification of growth promoting activity from 5.1 litres of bovine semi-skimmed milk

	Volume (ml)	Total protein (mg)	Total act.* (units)	Spec.act. (units/mg)	Recovery (%) per step	Recovery (%) in total	Fold of purification per step	Fold of purification in total
Crude milk	5100	173,400	236,612	1.36	100	100	1	1
Acid extraction	3650	12,008	217,884	18.14	92.1	92.1	13.34	13.34
$(NH_4)_2SO_4$ salt out	1605	4,397	88,789	20.19	40.1	37.5	1.11	14.85
CM-sepharose chromatography	165	27.15	38,975	1,435.5	46.1	16.5	74.49	1,055.51
Hydrophobic interaction chromatography	73.5	2.31	28,998	12,553.2	74.4	12.26	8.75	9,230.29
Reversed phase HPLC (C4 column)	11.05	0.021	8,010	381,428.6	27.6	3.4	30.38	280,462.2
Reversed phase HPLC (C18 column)	0.48	0.015	702	46,800	8.8	0.3	_	34,411.76

FIG. 6

INTERNATIONAL SEARCH REPORT

Intr onal Application No
PCT/GB 96/02658

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 A23K1/16 A23L1/305 A23C9/12 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A23K A23L A23C A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 457 565 A (MORINAGA MILK INDUSTRY CO LTD ;IWASE COSFA CO LTD (JP)) 21 November 1991 see the whole document ---	1-3, 13-20
A	DATABASE WPI Section Ch, Week 9435 Derwent Publications Ltd., London, GB; Class B04, AN 94-283276 XP002013699 & JP 06 211 689 A (KANEBO LTD) , 2 August 1994 see abstract --- -/--	1-20

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 March 1997	24. 03 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Groenendijk, M

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/GB 96/02658

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 9201 Derwent Publications Ltd., London, GB; Class B04, AN 92-002669 XP002013698 & JP 03 255 095 A (KANEBO KK) , 13 November 1991 see abstract	1-20
P,X	BIOCHEM.SOC.TRANS., vol. 24, no. 3, 1996, page 342s XP000645809 LIU Q-M E.A.: "A growth factor activity in bovine milk" see the whole document	1-20

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

ernational application No.
PCT/GB 96/02658

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 1-5,13-20
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

See annex

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/GB 96/ 02658

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Annex to supplemental sheet B:SA145040

The scope of the claims 1-5 is unclear and speculative. The claims 1-3 lack any indication concerning the (minimal) size of the peptide, e.g. even include dipeptides. Moreover expressions like "substantial identical" (claim 1) and "homologue" (claims 2 and 3) cannot be considered to be clear and concise definitions of patentable subject-matter, especially not in combination with an insufficient structural definition (Art.6 PCT).

Furthermore the available experimental data actually only comprise a very small part of the compounds claimed, which part is moreover not evenly distributed over the whole claimed area. Therefore the claims can also not be considered to represent a permissible generalisation which is fairly based on experimental evidence, that is, they are also not adequately supported by the description (Art.6 PCT).

Therefore a meaningful and economically feasible search could not encompass the complete subject-matter of the claims. Consequently the search has been limited to the use of the actually synthesised compounds and (closely) related analogs, that is the compounds encompassed by the claims 6-12 having a length from 9-31 amino acids, and extended to analogous compounds originating from the other species mentioned in the description. (Art.17(2)(a)(ii) PCT).

INTERNATIONAL SEARCH REPORT

information on patent family members

Inte ional Application No
PCT/GB 96/02658

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0457565 A	21-11-91	JP 4026604 A	29-01-92
		JP 4026605 A	29-01-92
		US 5314873 A	24-05-94